THE FRACTIONATION OF THE NITROGEN COMPOUNDS OF WHEAT FLOUR ON SEPHADEX COLUMNS*

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Introduction

Bagdasarian *et al.* (1964) have discussed the difficulties in separating compounds of low molecular weight from proteins, and the advantages of using a volatile solvent, such as phenol–acetic acid–water (1 : 1 : 1, w/v/v) (referred to as solvent A in the remainder of this paper), to achieve this separation. Trichloroacetic acid is also efficient in effecting this separation (Bagdasarian *et al.* 1964), but it dissolves glycoproteins (Bell 1963; Stenzel *et al.* 1964; Jennings and Watt 1967) and some of the wheat flour proteins (Bell 1963) and is difficult to remove from solutions.

Solvent A is an excellent solvent for wheat flour proteins (Jennings and Watt 1967). Also, hydrolysis of peptide bonds, sulphydryl–disulphide interchange, oxidation of sulphydryl groups, or other chemical changes are unlikely to occur when this solvent is used (Bagdasarian *et al.* 1964; Brattsten, Synge, and Watt 1965). This paper describes investigations into its use for the separation of wheat flour proteins from compounds of low molecular weight. The fractionation of the products of hydrolytic degradation of these proteins, using the chromatographic procedures of Bagdasarian *et al.* (1964) and Carnegie (1965*a*), is also described.

Materials and Methods

Sephadex G-25 and G-75 gels were obtained from Pharmacia, Uppsala, Sweden. The purified preparation of deoxyribonucleic acid of high molecular weight prepared from calf thymus and the tobacco mosaic virus were kindly supplied by Dr. J. F. Jackson and Dr. R. J. Best, respectively, of this Department. The commercial preparation of yeast ribonucleic acid was obtained from Hopkin and Williams Ltd., Essex, England. Phenol and glacial acetic acid were redistilled before use. All other compounds and reagents were of AR grade.

The Sephadex gels were packed into columns made entirely of glass to avoid the use of rubber or polyvinyl chloride, which are degraded by mixtures of phenol, acetic acid, and water.

Grain of *Triticum vulgare* cv. Gabo, grown in 1960, was milled to approximately 70% extraction on a Buhler laboratory mill. The endosperm was dissected from grain of *T. vulgare* cv. Gabo and *T. durum* cv. Dural, both harvested 19 days after the mean date of flowering in 1963, by the procedure of Jennings and Morton (1963*a*).

(i) *Preparation of Extracts of Flour.*—The flour was suspended in solvent A and treated ultrasonically for 30 sec in an ice–water bath with a Branson Sonifier (Model LS-75; Branson Instruments Inc., Stamford, Connecticut, U.S.A.). The suspension was centrifuged at 3000 *g*

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for 15 min at room temperature. The residue was re-extracted twice by the same procedure. The combined extracts contained 98·2% of the total nitrogen and 18·6% of the total dry matter.

(ii) *Preparation of Proteins from Endosperm.*—The endosperm was fractionated by the procedure of Morton and Raison (1964) which involves differential centrifugation of a homogenate of the endosperm. A portion of the material, which sedimented at 100 *g*, was extracted with solvent A containing 0·2M NaBr (to be referred to as solvent B). Four volumes of dioxan were added to the extract (Jennings and Watt 1967) and the precipitate formed was washed with ether and dried in air.

(iii) *Hydrolyses of Proteins.*—The dried precipitates derived from endosperm were hydrolysed with 0·03N HCl (Tsung and Fraenkel-Conrat 1965) for 16 hr at 110°C in evacuated, sealed tubes. The ratio of dilute acid to sample was about 1000 : 1 (v/w). A portion of ovalbumin, prepared by the procedure of LaRosa (1927), was also hydrolysed. The hydrolysates were dried in a rotary film evaporator and dissolved in solvent A.

(iv) *Monitoring of Eluted Fractions.*—Portions (0·01 ml) of the eluted fractions were transferred to strips of Whatman No. 1 paper. The strips were treated by conventional staining procedures to show the distribution of the components between the fractions. To detect rather small amounts of some components, larger portions were evaporated to dryness in the depressions of a glazed tile at 60–80°C. The residues were dissolved in 0·05 ml of water or dilute ammonia and transferred to the paper strips.

Proteins and nitrogen compounds of high molecular weight were detected by staining the strips in 0·07% (w/v) nigrosin in 7% (v/v) acetic acid for 2 min before washing in 7% acetic acid. The strips were washed in ethanol before colour development to remove the residual amounts of phenol which caused non-specific staining.

Amino acids and peptides were detected by spraying the strips with ninhydrin reagent (Stepka 1957), the colours being stabilized with copper nitrate (Mabry and Todd 1963). Unidimensional chromatography was carried out by the procedure of Carnegie (1965*a*). Individual fractions were analysed by two-dimensional chromatography, using the solvent systems, n-butanol–acetic acid–water (4 : 1 : 5 by vol.) (Partridge 1948) and n-butanol–methylethylketone–water–cyclohexylamine (10 : 10 : 5 : 2 by vol.) (Hardy, Holland, and Naylor 1955).

Reducing sugars were detected by the procedure recommended by Block, Durram, and Zweig (1958). When sodium bromide was present in the eluted fractions, or it was likely that polysaccharides were present, two other procedures were used. Portions (0·25 ml) of the eluted fractions were mixed with 0·5 ml of 80% (v/v) H_2SO_4 or with 0·2% (w/v) anthrone in 80% H_2SO_4 (Scott and Melvin 1953) and heated in a boiling water-bath for 3 min.

Nucleotides and nucleic acids were detected by their absorption of ultraviolet light, nucleic acids also being detected by their reaction with gallocyanine (Einarson 1932; Beswick 1958).

Results

(i) *Efficiency of Elution.*—A saturated solution of nigrosin in solvent A was washed through the Sephadex columns immediately after elution of the compounds of low molecular weight. The lack of staining in the gel indicated that the proteins had been eluted completely.

(ii) *Effect of Sodium Bromide on Flow Rates.*—Flow rates of solvents A and B through Sephadex G-75 were the same. When a 0·5-ml portion of the extract of flour in solvent A was loaded on to a Sephadex G-75 column (12·7 cm high) in this solvent, the flow rate immediately slowed down by a factor of about four. The protein did not emerge in the appropriate effluent fractions and a distinct haziness was apparent in the first 6 cm of the gel, indicating that the protein may have been adsorbed in that region. The eluting solvent was changed to solvent B. The haziness in the upper portion of the column disappeared on contact with the sodium bromide. The first

fraction to contain bromide also showed the first positive test for protein. The protein was eluted in a volume of 3 ml. After the sodium bromide had commenced to emerge from the column, the flow rate increased markedly. Subsequently, 0·2M sodium bromide was included in, or added to, all extracts of flour proteins before loading on to Sephadex columns and was also included in the eluting solutions. Under these conditions, flow rates were not decreased by proteins.

The acid hydrolysates (dissolved in solvent A), were readily eluted from the Sephadex columns without any effect on flow rate. Sodium bromide had no effect on the rate of elution of this material.

(iii) *Separation of Known Compounds.*—The tobacco mosaic virus, deoxyribonucleic acid, glycine, glutamic acid, tyrosine, glucose, and adenylic acid were eluted by solvent B from the columns without retardation due to adsorption (Porath 1962; Bagdasarian *et al.* 1964; Carnegie 1965*a*, 1965*b*). The commercial preparation of yeast ribonucleic acid contained some material of low molecular weight.

(iv) *Fractionation of Flour Extracts.*—The compounds of high and low molecular weight were readily separated from one another on Sephadex G-75. The fraction of high molecular weight appeared to contain only protein whereas the other fraction contained only amino acids (and peptides, if present) and saccharide material.

(v) *Fractionation of Protein Hydrolysates.*—The hydrolysates, dissolved in solvent A, were eluted through Sephadex G-25 in the same solvent. Paper chromatography of the compounds of low molecular weight derived from cv. Gabo showed that most of the amino acid present was aspartic acid. Small amounts of lysine or arginine or both, and glutamic acid, glycine, and serine were also detected. The amino acids derived from cv. Dural and ovalbumin also consisted mostly of aspartic acid, with small amounts of glutamic acid, glycine, and serine.

The fractions from each hydrolysate which contained the greatest amounts of the compounds of high molecular weight were combined and eluted through Sephadex G-75 in solvent A. The fractions containing the material of high molecular weight were examined by gel electrophoresis, using the techniques described by Jennings (1968). Although several different buffer systems were used, this material could not be resolved satisfactorily into zones but migrated freely through the gels as a completely heterogeneous mixture.

Discussion

Our results with extracts of wheat flour and with other materials confirm the conclusions of Bagdasarian *et al.* (1964) and Carnegie (1965*a*, 1965*b*) that good separations of compounds of high and low molecular weight can be obtained on Sephadex G-25 and G-75 in solvent A. Thus, this procedure should provide a satisfactory alternative to treatment with trichloroacetic acid (Bagdasarian *et al.* 1964).

The decrease in flow rate observed by Bagdasarian *et al.* (1964) during the elution of leaf proteins from Sephadex G-75 in solvent A was attributed to the formation of a thixotropic gel by the proteins after their separation from the compounds of low molecular weight.

A similar decrease in flow rate was observed with the wheat flour proteins. Since sodium bromide overcame this effect, it is likely that the proteins, after separation from the charged compounds of low molecular weight, aggregated, or interacted with the few weak negative charges in the gel (Porath 1962), through electrostatic bonds. Mixtures of phenol, acetic acid, and water have low dielectric constants (Singer 1962; Brattsten, Synge, and Watt 1965) and thus electrostatic interactions are enhanced in these solvents. Brattsten, Synge, and Watt (1964, 1965) and Jennings and Watt (1967) found that sodium bromide promotes the dissociation of protein–nucleic acid complexes and the solubility of nucleic acids in phenol–acetic acid–water (2 : 1 : 1 or 1 : 1 : 1, w/v/v). Presumably, the small molecular sizes and increased numbers of carboxyl groups (due to hydrolysis of amide groups) of the hydrolytic products of the wheat endosperm proteins prevent the formation of complexes which impede free flow of the eluting solvent and migration of the constituent molecules.

Since the reduced flow rate and the immobility of the protein molecules seems to be due to separation of the compounds of low molecular weight, this may indicate that the latter compounds can influence the rheological properties of doughs.

The failure to resolve the hydrolytic products of high molecular weight into zones by gel electrophoresis may have been due to the random removal of amide groups from the glutamine residues, thus increasing the degree of electrophoretic heterogeneity.

The preferential release of aspartic acid from a mixture of proteins of high glutamine and proline contents (Jennings and Morton 1963*b*), as well as from proteins of more typical amino acid compositions (Tsung and Fraenkel-Conrat 1965), shows that this procedure could be useful in other studies involving the controlled chemical degradation of wheat proteins. Since significant amounts of peptides of low molecular weight were not liberated from the wheat proteins, it is concluded that the aspartic acid residues tend to occur at widely separated points in the polypeptide chains.

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